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DETERMINATION OF DIAMINES AND POLYAMINES IN TISSUES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

The 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) derivatives of 1,2-diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane (putrescine), spermidine and spermine have been separated by high-pressure liquid chromatography on a Micropak CN-10 column using a programmed solvent gradient elution. The column eluate is monitored by a fluorescence detector. This method has been used to determine the levels of putrescine, spermidine and spermine in various tissues of rats and in L 1210 leukemic cells of mice grown in culture. The technique is sufficiently sensitive to detect *ca.* 40 pmoles of putrescine and *ca.* 20 pmoles of spermidine and spermine, is quite specific and can be performed rapidly.

INTRODUCTION

The polyamines spermidine and spermine and the diamine putrescine are distributed widely in animals and plants[1]. The exact biological role of these compounds has not been elucidated, but these amines and their biosynthetic enzymes are elevated in neoplastic tissue and in response to a stimulus which produces growth in normal tissues[2]. Therefore, it has been postulated that these amines play an important role in cellular growth and they may be necessary for the enhanced rates of deoxyribonucleic acid (DNA) replication in rapidly proliferating systems[3].

In order to investigate the role of these amines in growth, sensitive and specific analytical procedures are needed to study the fluctuations in the levels of these amines in tissues. Thus several procedures have been reported which involve various analytical techniques[4–13], and the relative merits of many of these methods were summarized in a recent report[13]. The most sensitive methods are probably those of Marton and Lee[11] and of Seiler and Wiechmann[12]. The use of the automatic amino acid analyzer as reported by Marton and Lee[11] is an excellent procedure for the determination of polyamines in samples of limited size such as animal tissues[11]. However, the high cost of the instrument precludes its use in many laboratories. The method of Seiler and

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Wiechmann[12] involves the formation of the 5-dimethylaminonaphthalene-1-sulfonyl (Dns) derivatives of the amines, separation on thin-layer chromatography (TLC) and measurement of the fluorescence of the Dns derivative of each amine, all of which is quite time consuming. Abdel-Monem and Ohno[14] reported the separation of the Dns derivatives of several di- and polyamines using high-pressure liquid chromatography (HPLC). In this paper we describe a sensitive and convenient method for the determination of polyamines in tissues by use of HPLC for the separation of the Dns derivatives obtained directly from the tissue extracts.

EXPERIMENTAL

Materials

Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride and Dns chloride (Dns–Cl) were purchased from Sigma (St. Louis, Mo., U.S.A.). [^{3}H]-Putrescine dihydrochloride, [^{3}H]-spermidine trihydrochloride, [^{3}H]-spermine tetrahydrochloride and [^{3}H]-toluene were obtained from New England Nuclear (Boston, Mass., U.S.A.). Permablend III and Triton X-100 were obtained from Packard (Downers Grove, Ill., U.S.A.). Fisher's medium for leukemic cells of mice (modified) (10X), horse serum, penicillin G and streptomycin were purchased from Grand Island Biological (Grand Island, N.Y., U.S.A.). Chloroform and isopropanol were A.C.S. grade and were distilled over calcium chloride before use. Triethylamine (Aldrich, Milwaukee, Wisc., U.S.A.) was distilled over potassium hydroxide and then over *p*-toluenesulfonyl chloride. Cyclohexane and methylene chloride were distilled in glass vessels (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). Other reagents and solvents were reagent grade. Silica gel GF plates (20 × 20 cm, 250 μm) were obtained from Analtech (Newark, Del., U.S.A.).

Preparation of tissues

A male Sprague–Dawley rat (350 g) was anesthetized with diethyl ether and the liver, prostate gland, spleen and brain were removed quickly and chilled in an ice-cooled solution of 0.15 *N* sodium chloride. The tissues were then dried with blotting paper, weighed and homogenized in three volumes of 0.4 *N* perchloric acid using a glass tissue grinder (Ace Glass, Vineland, N.J., U.S.A.). The tissue homogenates were centrifuged at 25,000 *g* for 30 min at 2°. The supernatant was stored in plastic tubes at −20° until required for the analysis.

L 1210 cells were suspended in Fischer's medium for leukemic cells containing 10% of horse serum, 13.4 m*M* sodium bicarbonate, 50 units/ml of penicillin G and 50 μg/ml of streptomycin. Aliquot portions of the cell suspension containing $1–2 \cdot 10^6$ cells were centrifuged at 1000 *g* for 5 min at room temperature. The resulting pellet was re-suspended in a 300 μl of 0.3 *N* perchloric acid and then centrifuged. The supernatant was stored at −20° until required for analysis.

Dansylation reaction

Disposable glass test-tubes (16 × 125 mm) were used for this reaction. The following volumes of each of the above tissue extracts were adjusted, if necessary, to 100 μl with 0.3 *N* perchloric acid and then diluted with 100 μl of water: prostate, 5 μl; liver, 25 μl; spleen, 15 μl; brain, 53 μl; and L 1210 cells, 100 μl. Standard solu-

tions for the calibration graph were prepared by mixing 100 μl of one of five standard solutions of polyamines, which contained various concentrations of putrescine, spermidine and spermine in water, with 100 μl of 0.3 *N* perchloric acid. The following solutions were added to each tube: 100 μl of a saturated solution (room temperature) of sodium carbonate; 20 μl of a 0.2 m*M* solution of 1.2-diaminoethane dihydrochloride (if appropriate); and 600 μl of a solution of 7 mg/ml of Dns–Cl in acetone. The tubes were stoppered with a plastic cap and shaken gently overnight at room temperature in the dark. The tubes were then immersed in a water bath at 40° and the liquid in each tube was evaporated by means of a stream of nitrogen. The residue in each tube was mixed vigorously for 20 sec with a vortex mixer with 0.5 ml of water and 5 ml of benzene. The tubes were centrifuged at 1000 *g* for 2 min and the benzene layer was transferred to a 12-ml conical centrifuge tube. The tubes were then immersed in a water bath at 40° and the benzene was evaporated by means of a stream of nitrogen. The residue was stored at −20° for no more than 4 days. In order to accelerate the process of drying with nitrogen, we used a manifold which could accommodate 15 tubes simultaneously.

The recovery of the amines was determined by adding a solution containing $1–2 \cdot 10^5$ dpm of [^{3}H]-putrescine, -spermidine or -spermine to the tissue extracts. These samples were treated as described above, except that the benzene extract of the Dns derivatives was transferred to a glass counting vial and then evaporated. A 5-ml aliquot portion of a cocktail of 5.5 g of Permablend III in 1 l of toluene was added to each vial, and the radioactivity was measured by use of a Beckman LS-150 liquid scintillation system. The total amount of tritium added to each reaction was determined using a cocktail of 5.5 g of Permablend III dissolved in 667 ml of toluene and 333 ml of Triton X-100. The counting efficiency was determined using a standard solution of [^{3}H]-toluene.

Chromatographic analyses

HPLC. HPLC was performed on a system composed of a U6K injector, two 6000 solvent delivery systems and 660 solvent programmer (Waters Assoc., Milford, Mass., U.S.A.), a LDC Model 1209 fluoroMonitor (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) and a two-channel strip chart recorder (Honeywell, Fort Washington, Pa., U.S.A.). The separation of the Dns polyamines was carried out on a Micropak CN-10 column (25 × 2.5 mm; Varian, Palo Alto, Calif., U.S.A.) with a solvent composed of cyclohexane–isopropanol (49:1) as solvent A and cyclohexane–methylene chloride–isopropanol (21:3:1) as solvent B. The residues of crude Dns derivatives obtained above were dissolved in 50 μl of methylene chloride and 5–10 μl of the resulting solution was injected into the system. The sample was eluted in the isocratic mode with solvent A for 5 min, and then with a programmed solvent-gradient elution using the concave gradient curve number 7. The gradient changed from 100% of solvent A to 100% of solvent B in 15 min at a flow-rate of 3 ml/min. The maximum pressure developed during the elution was *ca.* 4500 p.s.i. Each sample was eluted in a total of 22 min, and then the column was allowed to re-equilibrate with solvent A for 3 min before a second sample was injected.

In selected samples, the purity of the column eluates, corresponding to the fluorescent peak for each of the Dns polyamines, was determined. Each fraction was concentrated and the residue was examined by HPLC using a column of Corasil II

(120 cm × 2.2 mm I.D., Waters Assoc.) and isocratic elution with chloroform–triethylamine (50:1) at a flow-rate of 0.5 ml/min.

TLC. The residues obtained by concentration of the above column eluates were also examined by two-dimensional TLC on a silica gel GF plate with chloroform–triethylamine (25:2) in one dimension and ethyl acetate–cyclohexane (1:1) in a second dimension.

Analysis of data

The areas of the peaks of interest were estimated by the triangulation method. The ratios of the peaks for each of the amines to that of the internal standard were calculated. The correlation graphs were obtained by regression analysis in order to determine the line of best fit to the data points.

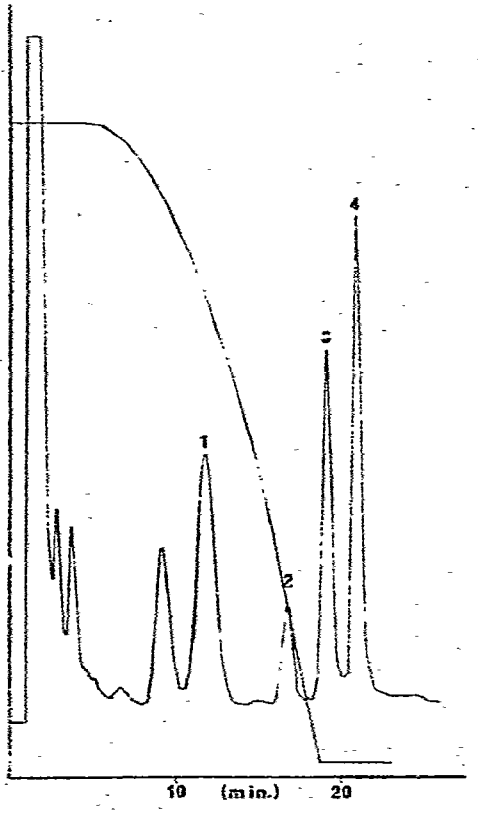

Fig. 1. HPLC separation of the Dns derivatives of a standard solution of: (1) 1,2-diaminoethane (internal standard); (2) putrescine; (3) spermidine and (4) spermine. Column, Micropak CN-10 (25 × 2.5 mm) (Varian Aerograph). Detector, fluoroMonitor (LCD). Solvent: isocratic elution with cyclohexane–isopropanol (49:1) for 5 min and then programmed gradient elution using concave curve number 7 (Waters 650 solvent programmer), a second solvent of Cyclohexane–methylene chloride–isopropanol (21:3:1) and a gradient time of 15 min; flow-rate, 3-ml/min (4500 p.s.i. initially). Temperature, ambient.

RESULTS

A typical separation produced by HPLC of the Dns derivatives of a mixture of 1,2-diaminoethane (internal standard), putrescine, spermidine and spermine is shown in Fig. 1. Dns ammonia and other fluorescent by-products of the dansylation reaction were eluted within 9 min of the injection, providing an excellent separation between the Dns ammonia peak and the 1,2-diaminoethane peak. The Dns derivative of 1,3-diaminopropane can also be separated successfully from the other Dns derivatives using this system as it was eluted between 1,2-diaminoethane and putrescine. The fractions of the column eluate corresponding to the four amines (peaks 1–4 in Fig. 1) were collected separately and evaporated by use of a stream of nitrogen. The residues were examined by HPLC with a column of Corasil II and chloroform–triethylamine (50:1) as solvent, and by two-dimensional TLC on silica gel with chloroform–triethylamine (25:2) as solvent in one dimension and ethyl acetate–cyclohexane (1:1) in the second dimension. These two tests revealed no fluorescent impurities in any of the four peaks.

Fig. 2 illustrates the linear relation between the amount of amine added and the ratio of the area of its peak to that of the internal standard 1,2-diaminoethane. The correlation coefficients, r, were calculated by regression analysis and found to be 0.966 for putrescine, 0.994 for spermidine and 0.997 for spermine.

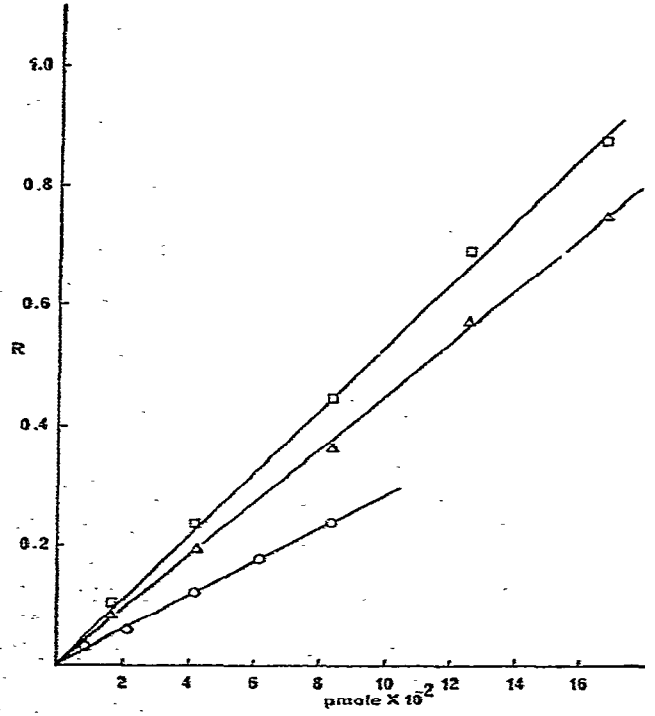

Fig. 2. Correlation graphs for the determination of the polyamines. R = Ratio of the area of the amine peak to the area of the internal standard peak. ●—●, Putrescine; △—△, spermidine; and □—□, spermine.

This chromatographic procedure was used to determine the concentration of putrescine, spermidine and spermine in several tissues of rats and in L 1210 leukemic cells of mice grown in culture. The chromatograms obtained from the Dns derivatives of perchloric acid extracts of the four tissues and the cells had peaks with the same retention volumes as putrescine, spermidine and spermine. Chromatograms obtained from extracts of liver and of prostate, to which 1,2-diaminoethane had been added as an internal standard, are shown in Figs. 3 and 4. The chromatogram of the Dns derivatives of the extracts of spleen, brain and L 1210 cells were similar and are not shown. The ratios of the area of the peaks produced by putrescine, spermidine and spermine to that of the internal standard were calculated. The levels of the amines in the extracts were then determined from the correlation graph (Fig. 2). The concentrations of the amines in the tissues and in the cells are listed in Table I. The peaks

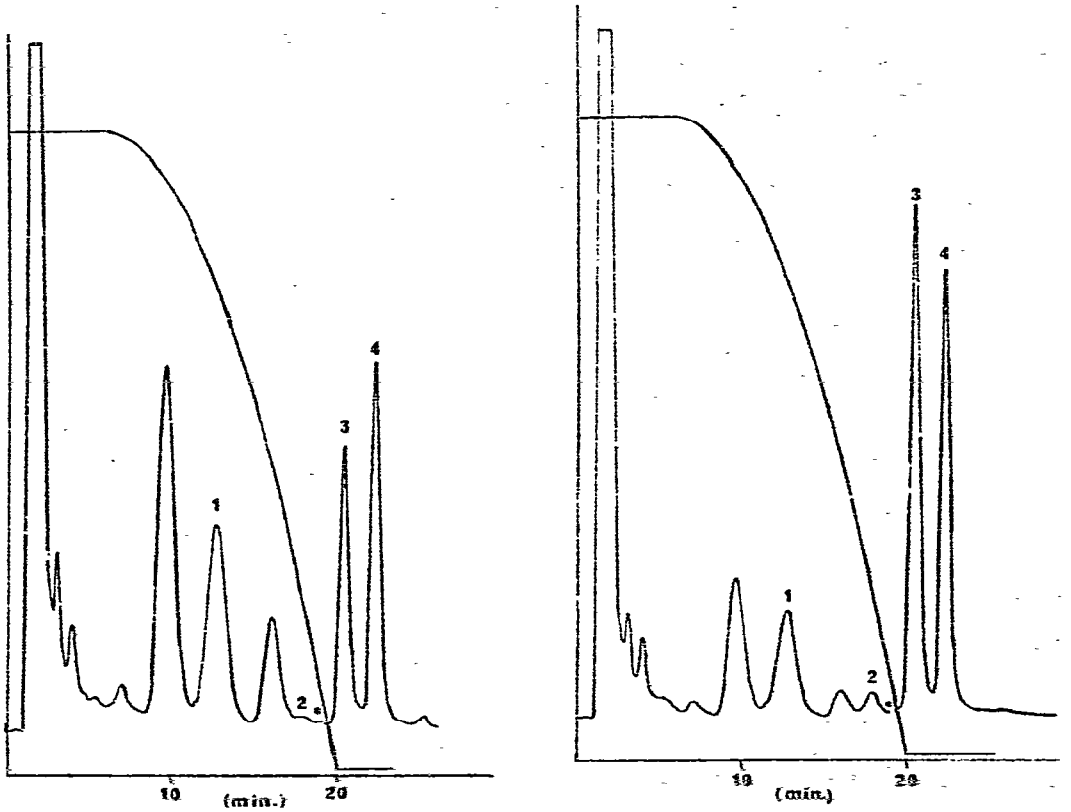

Fig. 3. HPLC separation of the Dns derivatives obtained from the rat liver extract to which 1,2-diaminoethane (internal standard) had been added. The separation conditions were similar to those described for Fig. 1. The sensitivity of the fluoroMonitor was reduced by a factor of 2 at the point marked with an asterisk. Peaks: 1 = 1,2-diaminoethane (internal standard); 2 = putrescine; 3 = spermidine; and 4 = spermine.

Fig. 4. HPLC separation of the Dns derivatives obtained from the rat prostate extract to which 1,2-diaminoethane (internal standard) had been added. Peaks: 1 = 1,2-diaminoethane (internal standard); 2 = putrescine; 3 = spermidine; and 4 = spermine. Other details as in Fig. 3.

produced by putrescine from the extracts of liver and brain were too small to quantitate. The values in Table I agree fairly well with those published for some of these tissues[15].

TABLE I

CONCENTRATION OF DI- AND POLYAMINES IN SOME TISSUES OF RATS AND IN L 1210 LEUKEMIC CELLS OF MICE

Tissue	*Putrescine*	*Spermidine*	*Spermine*
Prostate*	337 ± 19	7635 ± 327	5365 ± 103
Liver*	—	454 ± 14	467 ± 9
Spleen*	28 ± 3	1061 ± 18	839 ± 23
Brain*	—	356 ± 16	236 ± 4
L 1210 cells**	24 ± 6	267 ± 20	65 ± 6

* Four aliquots of extracts of prostate, liver and spleen and three aliquots of extract of brain from one rat were analyzed. The concentrations are in pmoles/mg wet weight (mean ± S.D.).

** Extracts of L 1210 cells from three separate culture tubes were analyzed. Concentrations are in pmoles/mg DNA (mean ± S.D.).

The percentage recoveries (mean ± S.D.) of the Dns derivatives of the amines from the tissues, from the L 1210 cells and from the standard solutions for the calibration graph were as follows: putrescine, 81 ± 3; spermidine, 73 ± 3; and spermine, 63 ± 3. The recovery of spermine from the extract of the prostate gland was 77 ± 2%, which is higher than for the other tissues, and was not included in the calculations of the mean percentage recovery given above. The data in Table I were corrected for this difference.

The extracts were processed without an added internal standard and the results were similar to those for spleen shown in Fig. 5. No peaks were found with the same retention volume as that of Dns 1,2-diaminoethane. In contrast, peaks with the same retention volume as Dns 1,3-diaminopropane were found in the extracts of all of the tissues and of the cells. The fraction of the column eluate corresponding to this peak was collected, concentrated and examined by HPLC on a column of Corasil II and by two-dimensional TLC on silica gel. These studies indicated that this fluorescent substance was not Dns 1,3-diaminopropane; however, no attempt was made to identify it. This compound was present in very low concentrations relative to the amount of 1,3-diaminopropane added as an internal standard in the extract of L 1210 cells, so 1,3-diaminopropane could be used as an internal standard for these cells. But, the concentration of the compound in the extracts of the tissues was too large to permit use of 1,3-diaminopropane as an internal standard without incurring a large error.

The fractions of the column eluate corresponding to Dns 1,2-diaminoethane, putrescine, spermidine and spermine were collected separately for all of the tissues and the cells. The fractions were concentrated and the residues were examined by HPLC on a column of Corasil II and by two-dimensional TLC on silica gel as described above. None of the amines was contaminated by more than 0.5% of other fluorescent compounds.

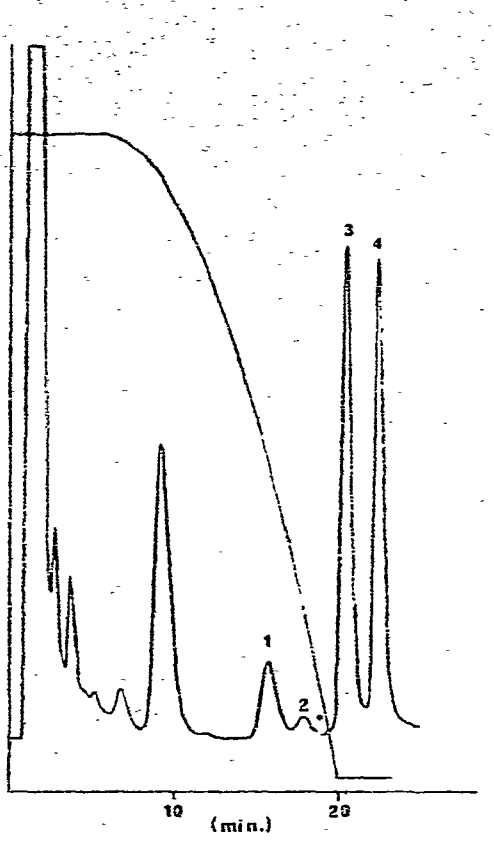

Fig. 5. HPLC separation of the Dns derivatives obtained from the rat spleen. Peaks: 1 = unknown constituent from tissues; 2 = putrescine; 3 = spermidine; and 4 = spermine. Other details as in Fig. 3.

CONCLUSIONS

We have developed a sensitive method for the determination of polyamines in tissues. The sensitivity of our method is probably equal to that of the method using an automated high-pressure amino acid analyzer which was recently described by Marton and Lee[11]. The advantages of our method are the short time for analysis using HPLC, and the fact that the instruments for this procedure are available in many laboratories. The method described in this report was used routinely in our laboratories for the analysis of hundreds of samples of L 1210 cells grown in culture and found to be reliable and convenient.

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